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Evolution

Extensive mitochondrial diversity within a single Amerindian tribe

(population genetics/molecular anthropology/Pacific Northwest/human evolution)

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ABSTRACT **Sequencing of a 360-nucleotide segment of the mitochondrial control region for 63 individuals from an Amerindian tribe, the Nuu-Chah-Nulth of the Pacific Northwest, revealed the existence of 28 lineages defined by 26 variable positions. This represents a substantial level of mitochondrial diversity for a small local population. Furthermore, the sequence diversity among these Nuu-Chah-Nulth lineages is >60% of the mitochondrial sequence diversity observed in major ethnic groups such as Japanese or sub-Saharan Africans. It was also observed that the majority of the mitochondrial lineages of the Nuu-Chah-Nulth fell into phylogenetic clusters. The magnitude of the sequence difference between the lineage clusters suggests that their origin predates the entry of humans into the Americas. Since a single Amerindian tribe can contain such extensive molecular diversity, it is unnecessary to presume that substantial genetic bottlenecks occurred during the formation of contemporary ethnic groups. In particular, these data do not support the concept of a dramatic founder effect during the peopling of the Americas.**

Genetic and archeological data support the hypothesis that, after initially evolving in Africa, modern humans rapidly expanded into Eurasia and subsequently into Australasia and the Americas (1–4). Thus, during this last major phase in human evolution, large geographic areas were rapidly colonized by migrating tribal groups. Analysis of molecular data has suggested that both the initial and subsequent migratory expansions of early human populations may have been accompanied by substantial reductions in genetic diversity (2, 5). In particular, the distribution of mitochondrial DNA variants in Amerindians has been interpreted as evidence for a dramatic bottleneck, which occurred during the peopling of the Americas (6, 7). These interpretations imply that small tribal groups—the primary demographic units of early human populations—contain only limited amounts of molecular diversity. However, such a conclusion runs counter to the observation that, for standard genetic markers, the amount of genetic differentiation within tribes represents an appreciable fraction of the genetic variability contained within continental populations (8).

To evaluate how much molecular diversity can be maintained within tribal populations, we have carried out a detailed study of mitochondrial diversity within a single tribe by determining the distribution of mitochondrial DNA sequences within the Nuu-Chah-Nulth (Nootka), a Wakashan-speaking group of the Pacific Northwest. The rapid rate of sequence divergence of mitochondrial DNA makes it suitable for the analysis of short-term evolutionary phenomena, while the maternal mode of inheritance allows the evolutionary relationships between lineages to be defined in terms of their phylogenetic divergence, without the ambiguities caused by recombination (9). Since the mitochondrial control region accumulates substitutions at a much faster rate than other regions of the molecule (10, 11), we increased the resolution of the study by enzymatically amplifying and directly sequencing the first 360 nucleotides of this DNA segment.‡ This region has been shown to be informative in detecting sequence divergence within other human populations (12, 13). DNA for the study was extracted from frozen serum samples selected to represent the geographic subdivisions within the Nuu-Chah-Nulth. The results were contrasted with the available data on sequence diversity in much larger regional populations (Japanese) and continental populations (sub-Saharan Africans). A phylogenetic analysis defined a molecular genealogy, in which the presence of lineage clusters suggests considerable heterogeneity in lineage ancestry, indicating that a considerable amount of mitochondrial diversity was introduced into the New World at the time of initial colonization.

MATERIALS AND METHODS

Population Sample. The Nuu-Chah-Nulth (Nootka) are a group of Wakashan speakers that comprise 14 bands located on the western coast of Vancouver Island, plus 1 band on the Olympic Peninsula of Washington state. The archaeological record indicates cultural continuity in this area over the past 4000 years (14). Analysis of genetic markers (ABO, MNS, and Rh blood groups) indicated that individuals born before 1940 had <5% Caucasian admixture. As part of a biomedical study, the traditional band communities, numbering some 2000–2400 people, were surveyed between 1984 and 1986. Serum samples were collected from a large proportion (45%) of the population, and detailed genealogical information was collected for each band, along with basic demographic data. To determine the amount of mitochondrial variability, we selected 63 maternally unrelated individuals whose genealogy indicated Nuu-Chah-Nulth descent at least as far back as the late nineteenth century. These individuals were selected from 13 of the 14 contemporary bands. An additional 5 individuals, each known to be maternally related to 1 of the 63 independent individuals, were also included in the study as positive controls. This allowed an assessment of the reliability of DNA sequences determined from frozen serum samples, which represents an unusual source of material for sequence determination.

Since mitochondrial DNA is maternally inherited, the effective gene number is defined in terms of the number of breeding females, N_f, and fluctuations in this demographic parameter will influence the amount of mitochondrial diversity maintained in the population (15, 16). We determined that approximately two-thirds of the 963 Nuu-Chah-Nulth females in our survey were of child-bearing age (between 15 and 45 years old), giving an N_f of 600 for the contemporary population. Unfortunately, it is not clear how accurately this

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‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M75991–M76018).

figure reflects the long-term effective population size for mitochondrial lineages. Like most Amerindian populations, the Nuu-Chah-Nulth experienced substantial demographic fluctuations in the aftermath of European contact. This included an initial period of population decline, the amalgamation of some formerly distinct bands, and, most recently, a period of rapid population growth. Since the amount of mitochondrial variability is more likely to have been diminished by the initial population decline than enhanced by the contemporary demographic expansion, this estimate of an effective population of 600 females may represent an upper bound for the distribution of mitochondrial lineages.

DNA Extraction. Serum samples that had been frozen at −20°C for 4–6 years were used for DNA extraction. Since such samples contain relatively small amounts of DNA, control extracts that contained no serum were performed along with each set of extractions in order to detect any contamination of reagents or laboratory ware by extraneous human DNA (17). To 300-μl aliquots of serum an equal volume of 10 mM Tris (pH 8.0), 2 mM EDTA, and 10 mM NaCl containing dithiothreitol (BRI) at 2 mg/ml and proteinase K (Boehringer Mannheim) at 100 μg/ml was added. This solution was incubated for 2–6 hr at 37°C. The solution was extracted twice with water-saturated phenol (pH 8.0) and once with chloroform/isoamyl alcohol (24:1, vol/vol). The water phase was concentrated and purified by three washes of distilled water over Centricon-30 microconcentrators (Amicon).

Enzymatic Amplification and Sequencing. An initial reaction amplified ≈10% of the DNA extract in a 25-μl reaction volume, as described (18), using equal concentrations of an external primer pair: L15926 (5′-TCAAAGCTTACACCAGTCTTGTAAACC-3′) and H16498 (5′-CCTGAAGTAGGAACCAGATG-3′). The numbers in the primer designations identify the 3′ ends according to the reference sequence (19), while L and H designate the light and heavy strands, respectively. An aliquot of this reaction was then used to seed two asymmetric amplifications (20) using a 1:50 ratio of two internal primers: L15997 (5′-CACCATTAGCACCCAAAGCT-3′) and H16401 (5′-TGATTTCACGGAGGATGGTG-3′). These single-stranded amplification products were purified by Centricon-30 microconcentrators, and 7 μl of the retentate was sequenced by using the limiting primer from the second amplification. Use of both the Sequenase and Taquence sequencing kits (United States Biochemical) ensured that all substitutions would be detected without ambiguity on both strands. Reaction products were separated by electrophoresis through 6% polyacrylamide gels containing 7 M urea. Gels were fixed in 5% glacial acetic acid/5% methanol for 30–60 min, dried, and exposed to Kodak XAR film for 12–48 hr. Sequences were read into the computer, aligned, and compared using the ESEE computer program (21).

Restriction Site Polymorphisms and Length Variants. The presence of the *Hin*cII restriction sites at nucleotides 7853 and 13,259 was analyzed by amplifying an aliquot of DNA by using the four primers L7773 (5′-GACGCTCAGGAAATAGAAAC-3′), H8001 (5′-ATCGGGAGTACTACTCGATTGT-3′), L13232 (5′-CGCCCTTACACAAAATGACATCAA-3′), and H13393 (5′-ATTTTTCGAATATCTTGTTC-3′). Ten microliters of the amplification reaction was mixed with 10 μl of 2× restriction buffer and digested with 5 units of *Hin*cII (United States Biochemical) for 2 hr. Half the reaction was analyzed by gel electrophoresis in 40 mM Tris acetate through a 4% SeaPlaque (FMC) agarose gel. The conserved *Hin*cII site at position 7997 served as an internal control for the restriction digest. When a restriction site proved to be absent, the intact band was cut out of the agarose gel and melted in 100 μl of water, and 1 μl was used to seed a second amplification using unbalanced priming to generate single-stranded DNA for sequence determination (20). A 9-base-pair (bp) deletion in region V was detected by amplifying an aliquot of DNA with the primers L08215 (5′-ATGCTAAGTTAGCTTTACAG-3′) and H08297 (5′-ACAGTTTCATGCCCATCGTC-3′) and electrophoresing the products on a 5% agarose gel, as described (22). When a length variant was detected, single-stranded DNA was prepared and sequenced, as described above, to confirm the presence of the 9-bp deletion.

Phylogenetic Analyses. Phylogenetic trees based on the 360 nucleotides from position 16,024 to position 16,383 were constructed by the maximum likelihood algorithm (23), as well as by maximum parsimony and, in the latter case evaluated by bootstrap analysis (24), using the PHYLIP (23) and PAUP (25) computer packages.

RESULTS AND DISCUSSION

Sequence Diversity. The complete sequence of a 360-bp segment of the control region (positions 16,024–16,383 in the reference sequence) was determined for 63 individuals. Sequence comparison identified 28 mitochondrial lineages defined by 26 variable positions (Fig. 1). The most frequent lineage occurred in 9 individuals, whereas 13 lineages were observed only once, giving an estimated diversity value, *h* (26), of 95.4% and a probability of lineage identity of 6.1%. No contaminating sequences were observed in the negative controls, whereas the five positive controls had sequences that were identical to those individuals to whom they were maternally related. This confirmed the reliability of sequence determination by enzymatic amplification of DNA extracted from frozen serum samples. Our success in using frozen serum for DNA sequence determinations, as well as for restriction site polymorphisms, indicates that a dramatic increase in the amount of genetic information can be obtained

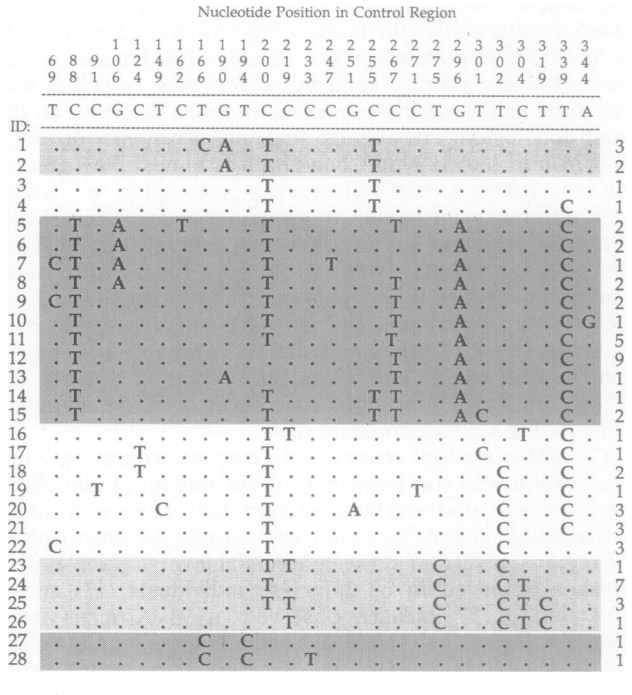

FIG. 1. Definition of 28 Nuu-Chah-Nulth mitochondrial lineages in terms of 26 variable positions within the control region, where position 69 corresponds to position 16,092 in the published human reference sequence (19). Dots indicate identity with the bases in the reference sequence, as defined by the first line. The number of individuals carrying a specific sequence is indicated in the right-hand column, and the four shaded regions highlight the lineages that fall into clusters I, II, III, and IV, respectively (see Fig. 2).

for many previously surveyed tribal populations by using existing frozen serum samples.

Since preliminary sequence data from additional individuals indicates the existence of at least another 3 or 4 lineages in the Nuu-Chah-Nulth, the 28 lineages defined in Fig. 1 represent a conservative indication of the number of lineages within this tribal population. By comparison, sequencing the same segment of the control region in 15 !Kung sampled from a much larger population revealed only nine lineages, defined by 19 positions (12). These observations can be used to estimate the relative effective size of the two populations, since the number of lineages observed in a sample of size n is given by the sum $\theta/\theta + \theta/(\theta + 1) + \ldots + \theta/(\theta + n - 1)$ (27), where $\theta = 2N_f \cdot \mu$, N_f is the effective population size in terms of the number of breeding females, and μ is the mutation rate. In this instance, where the same sequence is studied in both populations, the mutation rate can be treated as a constant. The !Kung sample gives a θ of 8.5, whereas the 28 lineages observed in the 63 Nuu-Chah-Nulth yields an estimated θ of 19, implying that the Nuu-Chah-Nulth have an effective population size that is 2.2 times larger than that of the !Kung, who number some 10,000 individuals (12). If the demographic structure of the two populations was roughly similar, this would imply a standing population of 22,000 for the Nuu-Chah-Nulth, which is an order of magnitude greater than their actual size. Alternatively, if the number of lineages observed in the two samples were to reflect the relative demographic size of the populations, only eight lineages would be expected in the sample of 63 Nuu-Chah-Nulth. These contrasts serve to emphasize the high levels of mitochondrial diversity maintained in this Amerindian tribe.

Use of these estimates of θ, in conjunction with our estimate that, assuming a generation of 20 years, the maximal rate of sequence divergence is 3.33% per 5000 generations (see below), suggests that the mitochondrial diversity observed in the Nuu-Chah-Nulth is consistent with an effective population size equivalent to 4000 females, whereas the diversity observed in the more numerous !Kung is equivalent to an effective population size of only 1800 females. Comparison of the average sequence divergence, which is proportional to the effective population size (26), leads to the same conclusion. The average sequence divergence of 1.49% ± 0.74% in the 63 Nuu-Chah-Nulth is almost 50% greater than the 1.09% ± 0.80% divergence observed in the !Kung. This difference is somewhat surprising because the !Kung live in an area that has been continuously inhabited for the past 100,000 years (1), whereas the Nuu-Chah-Nulth live in a much more recently occupied area. The demonstration that the effective population size of the Nuu-Chah-Nulth, estimated in terms of breeding females, is notably larger than their census population, while the reverse is true for the !Kung, argues for substantial differences in the demographic history of these two populations.

The mitochondrial sequence divergence within the Nuu-Chah-Nulth was also compared to the amount of mitochondrial diversity observed in populations that are several orders of magnitude larger. Two data sets permitted comparison at the regional level and at the continental level: a sample of 49 lineages observed in 62 Japanese individuals (13) and a collection of 57 lineages observed in 94 Africans from Namibia, Botswana, Tanzania, Zaire, Nigeria, and the Central African Republic (13, 28). This comparison had to be restricted to only 260 nucleotides because complete sequence data for all 134 lineages was only available between positions 16,124 and 16,384. Because the demographic structure of the three types of populations is not comparable and because the samples are far from random, the comparison was defined in terms of nucleotide differences between lineages, without regard to lineage frequency. The data indicate that the average number of sequence differences among the 28 Nuu-Chah-Nulth lineages amounts to 81% of the sequence differences observed among 49 Japanese lineages and 62% of the sequence differences among the 57 African lineages (Table 1). Thus, relative to a large regional population such as the Japanese, a remarkable amount of sequence diversity can be maintained within a single tribe. The observation that this Amerindian tribe has 62% of the sequence differences observed in 57 African lineages is even more striking, since the African data incorporates mitochondrial lineages from widely scattered localities throughout sub-Saharan Africa.

The observation of extensive sequence divergence between Nuu-Chah-Nulth lineages extends to the molecular level the finding that an appreciable proportion of human genetic variability is maintained within tribal populations (8). By contrast, the available data from surveys of restriction site polymorphisms in other vertebrate species indicate only moderate amounts of mitochondrial diversity within local populations (29). Even the increased resolution of direct sequence comparisons has so far failed to identify significant mitochondrial diversity within local populations. For example, in Panamint kangaroo rats (*Dipodomys panamintinus*) sequence diversity does not exceed more than 10 mitochondrial lineages within the local populations, which number many thousands (30). The low levels of sequence diversity observed within the species flocks of haplochromine fishes within the African Rift Lakes (31) is an even more striking example of restricted mitochondrial diversity within geographically contiguous populations. Thus, compared to other vertebrate species, local human populations may be characterized by relatively large amounts of mitochondrial diversity.

Phylogenetic Analysis. To gain a perspective of the dynamics of lineage evolution, we constructed a phylogeny for the 28 Nuu-Chah-Nulth lineages (Fig. 2) using Felsenstein's maximum likelihood algorithm (23). This phylogenetic tree agrees in all its major features with a maximum parsimony tree, whether constructed by the PHYLIP (23) or PAUP (25) phylogeny packages. Inspection of the maximum likelihood tree depicted in Fig. 2 suggested that the majority of lineages fell into four clusters. Bootstrap analysis (24) gave reasonable statistical support for clusters IV and II but somewhat weaker support for clusters I and III (Fig. 2). However, the evolutionary distinctiveness of cluster III, as well as of cluster IV, was independently corroborated by the distribution of two restriction sites (*Hin*cII sites at positions 7853 and 13,259) and a 9-bp deletion (22). Lineages 1–22 lack the 9-bp deletion but possess both *Hin*cII sites. By contrast, lineages 23–26 in cluster III lack the *Hin*cII site at position 13,259, though they resemble lineages 1–22 in lacking the 9-bp deletion and possessing the *Hin*cII site at position 7853. The absence of the *Hin*cII site at position 13,259 in cluster III was shown to be due to the same A to G transition at position 13,263 by sequencing the amplification product for one representative of each lineage within the cluster. Last, although lineages 27 and 28 in cluster IV possess both *Hin*cII sites, they are unique in possessing the 9-bp deletion. Sequence analysis confirmed that the 9-bp deletion observed in lineages 27 and 28 was identical to the deletion described by

Table 1. Mean pairwise sequence differences within three levels of human populations

Population sample	Sequence difference, %		
	Absolute	Relative to continent	Relative to region
Continental (sub-Saharan Africans)	3.16	100	132
Regional (Japanese)	2.40	76	100
Tribal (Nuu-Chah-Nulth)	1.95	62	81

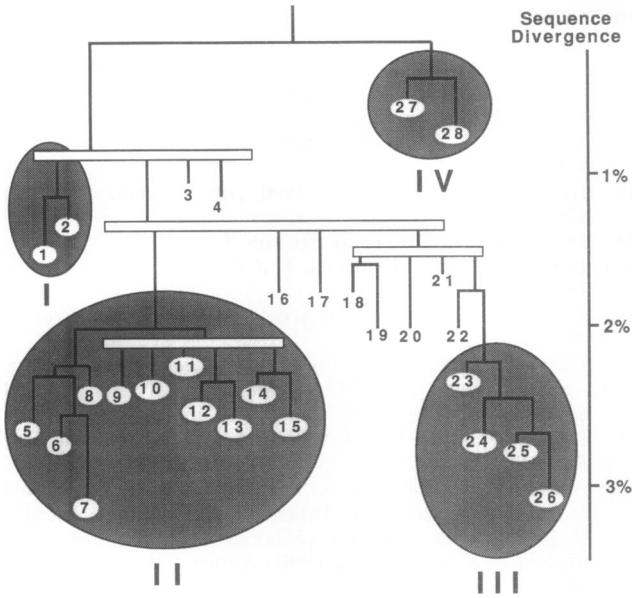

FIG. 2. A phylogenetic tree for 28 mitochondrial lineages in the Nuu-Chah-Nulth. The maximum likelihood phylogeny presented was estimated by using the PHYLIP package (23), assuming a 1:20 transversion-to-transition bias and using a !Kung sequence (!Kung sequence no. 2 in ref. 12) as an outgroup to root the tree. Nodes that fail to give statistically reliable estimates of branching order are indicated by open boxes. The data were also analyzed by maximum parsimony using the PHYLIP (23) and PAUP (25) packages. Both gave equivalent results that agreed in all major features with the maximum likelihood tree. Shaded regions illustrate the four lineage clusters for which 1000 bootstrap replications of the parsimony tree (24) gave the following levels of support: cluster IV, 86%; cluster II, 70%; cluster I, 57%; cluster III, 52%.

Wrischnik *et al.* (22). The distribution of these additional mitochondrial polymorphisms, located outside the control region, thus corroborates the existence and delimitation of clusters III and IV as determined by the phylogenetic analysis.

The existence of well-defined lineage clusters in a single local population, which suggests heterogeneity in the ancestry of the mitochondrial lineages, is unusual in the sense that most previous surveys have found essentially monophyletic mitochondrial sequences within local populations (29). The impact of this ancestral heterogeneity on the distribution of sequence diversity is highlighted by the fact that the average pairwise sequence difference between lineage clusters (1.98% ± 0.44%; range 1.3–2.6%) is more than three times the amount of sequence difference within clusters (0.50% ± 0.22%; range 0.3–0.8%). Since restriction site polymorphisms identified a diverse set of mitochondrial lineages in highland New Guinea (32), it is possible that, unlike the local populations of other species, human tribal populations will contain heterogeneous clusters of mitochondrial lineages.

Age of Lineage Clusters. Comparison of the amount of sequence divergence between humans and the common chimpanzee for the 360-bp segment of the control region (33) allows a tentative estimate for the temporal divergence between the Nuu-Chah-Nulth lineage clusters. Estimates of the date when humans and chimpanzees diverged range from 4 million years, indicated by molecular work (34), to 9 million years, suggested by paleontological evidence (35, 36). To obtain a conservative estimate for the temporal divergence between the tribal lineages, we chose the most recent date for the chimpanzee–human divergence, as well the highest estimate of the transversion-to-transition ratio (1:30) reported for pairwise comparisons of sequences of the human control region (13). Applying these values to the substitutions observed in the 360-nucleotide stretch gives a maximum rate of evolution of 33% divergence per million years for this DNA segment. Applying this presumed evolutionary rate to the average sequence divergence between clusters gives minimum estimates of the ages of the lineage clusters (Table 2). Since these times range from 41,000 to 78,000 years ago with an average age of 60,000 years, they predate by a large margin the proposed dates for the entrance of humans into the Americas, which range from a conservative date of 15,000 years ago (37) to the more radical suggestion of 33,000 years ago (38). Our estimated divergence times are quite conservative because use of a slower evolutionary rate, or use of maximum sequence divergence rather than average sequence divergence, would yield much greater ages for the divergence between lineage clusters. Hence, we conclude that these lineage clusters originated well before humans entered the Americas. However, since the sequence differences observed within lineage clusters correspond to a divergence time of approximately 8000 to 15,000 years ago, we suggest that much of the lineage diversity within clusters reflects evolution that occurred within Amerindian populations.

Implications for the Peopling of the Americas. It seems reasonable to assume that, to a first approximation, the tribal groups that inhabited Beringia during the last glaciation and that eventually migrated to the Americas were not too dissimilar from the contemporary Nuu-Chah-Nulth in overall population size and underlying band structure. Consequently, the substantial mitochondrial divergence found within the Nuu-Chah-Nulth argues against the notion, based on low resolution restriction site analysis (6, 7), that the formation of the Amerindian ethnic group was accompanied by a substantial genetic bottleneck. Our conclusion that the founding tribal groups introduced considerable genetic variability is borne out by the demonstration that the Nuu-Chah-Nulth contain lineage clusters that predate the colonization of the Americas. Given the amount of temporal divergence between the clusters, a substantial amount of this ancestral sequence diversity must have been present in the populations that colonized the Americas during the last glaciation. Preliminary analyses of sequence data for the same mitochondrial segment from other Amerindian tribal groups indicate that the majority of tribes are as diverse as the Nuu-Chah-Nulth and that only a small subset of the lineages found in one tribe is shared with other tribes. Further detailed analyses of mitochondrial sequence variation in additional Amerindian tribal populations will not only determine the magnitude of mitochondrial diversity introduced by the original founders but will also allow a test of the "three wave" hypothesis advanced to explain the colonization of the New World (39).

CONCLUSIONS

This study of mitochondrial sequence diversity within a tribal population confirms at the molecular level the previous finding (8) that tribal groups can contain a substantial proportion (up to 60–80%) of the genetic variability that exists within much larger regional and continental populations. The

Table 2. Sequence differences and minimum times of evolutionary divergence between lineage clusters in the Nuu-Chah-Nulth

	I	II	III	IV
I	—	1.86	1.74	1.34
II	56,000	—	2.59	2.31
III	53,000	78,000	—	2.01
IV	41,000	70,000	61,000	—

Mean pairwise sequence differences, expressed as percents, are given above the diagonal, whereas minimum times of divergence between clusters, expressed in years, are below the diagonal.

results of the phylogenetic analysis indicate that much of the observed molecular diversity is due to evolutionary events that occurred well before the initial formation of the ancestral population from which the contemporary population is derived. Both the extensive sequence divergence and the number of lineages observed in the sample are consistent with an evolutionary effective population size for the tribe that is substantially larger than the effective population size predicted from the demographic census. This contrasts with the situation in most other vertebrate species, in which local populations have limited mitochondrial diversity (29, 30), and suggests that human tribes are the evolutionary equivalents of much larger populations—presumably because they are an integral part of a larger network of interacting populations. Hence, the colonization of a new geographic area by such tribal populations is unlikely to be accompanied by substantial genetic bottlenecks. Consequently, the migratory expansions of early human populations that led to the formation of major ethnic groups most likely did not result in substantial genetic differentiation between groups. In particular, the magnitude, and age, of the mitochondrial lineage diversity observed within the Nuu-Chah-Nulth argue against the hypothesis of a dramatic founder effect during the peopling of the Americas.

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